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Concepts in Imaging and Microscopy: Color Image Processing for Microscopy

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Abstract. Digital image analysis has long been applied to monochrome images from the light microscope. Using these techniques one can locate, measure, identify, and count the objects of interest in a microscope field of view. Since microscope images often contain color information of interest, it is now becoming more common to analyze the spectral content of images as well. Although most of the digital imaging techniques developed for monochrome microscopy work well with color images, there are a few considerations specific to the analysis of multispectral imagery. These include obtaining a properly balanced multicolor digital image, specifying the color components of the image, and compensating for imperfect separation among the color channels.

Introduction

In two-dimensional digital image processing for microscopy, images are commonly considered to have a single scalar gray level (brightness or optical density) that is a function of two spatial coordinates. With color imaging there are two or more gray levels per pixel, each corresponding to a different spectral band. When we process three spectral bands corresponding to the red, green, and blue colors to which the human visual system responds, we call it "color image processing." A tri-color image can be formed by separately sampling three spectral bands over the two spatial coordinates of an optical image. In this article we focus on tri-color image analysis, although the generalization to four or more colors is straightforward.

The legacy of human vision

The retina of the human eye is covered with photoreceptor cells that are functionally analogous to the sensor sites (picture elements, or "pixels") on the CCD chip in a television camera. These cells absorb light from the image formed on the retina by the lens and cornea, and they send nerve impulses to the brain. The photoreceptor cells are a mixture of two types, rods and cones. While the more sensitive rods provide night vision, the cones afford color vision at higher light levels.

The cones occur in three types, each having a different spectral sensitivity. They divide the visible portion of the electromagnetic spectrum into the three bands that we perceive as red, green, and blue (RGB). These, then, are the three primary colors of human vision. Figure 1 shows the sensitivity spectra of the three types of cones in the human visual system (Wald, 1964). Notice the significant degree of overlap.

Because human vision uses three color channels, the bulk of the effort in the development of electronic imaging hardware has been devoted to tri-color systems. This is particularly true for television cameras, image digitizers, display systems, and image printers. As a result, tri-color equipment is produced in high volume and is widely available at relatively low cost. Digital image analysis techniques can be applied to images having any number of primary color channels, but the tri-color model is often used, simply because of hardware availability. We focus here on tri-color imaging to illustrate the most important points. In practice, however, the nature of the problem should dictate how many color channels are used.

Color digital image analysis

A monochrome digital image can be thought of as a scalar function of two spatial dimensions (x, y). By extension, a color digital image can be viewed as a scalar function of three dimensions, two spatial and one spectral (x, y, λ). It is usually more convenient, however, to treat

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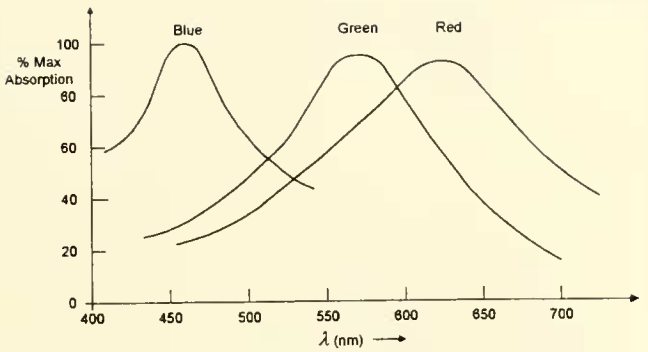

Figure 1. The spectral sensitivity of the cone photoreceptors in the human eye (after Wald, 1964).

it as a two-dimensional image having multiple gray levels (*e.g.*, red, green, and blue) at each pixel instead of only one. In other contexts it is more useful to consider it to be an overlay of monochrome digital images, each representing one of the primary colors. (We refer to these overlays as *color component images* or as the *color channels.*) The latter two viewpoints simplify the processing and analysis of color images because many of the techniques commonly applied to monochrome images can then be applied to color images with little modification.

Microscope image analysis

Three types of microscopic specimens exhibit color. Some specimens have color intrinsically. Examples are red blood cells and organic mineral samples. Other specimens take on color only as a result of staining or other chemical preparation prior to examination. A third category includes specimens given color by the optics of the microscope (polarization, birefringence, etc.).

In biomedical work, different chemical constituents of a specimen are often stained with absorbing or fluorescent dyes of different colors. In many applications the different structures are separable by color, and one wishes to visualize or measure these objects separately and in correct spatial relationship to one another.

If, for example, three chemical components of a specimen are stained, separately, with red, green, and blue fluorescent dyes, one can digitize and display a normal tri-color image of the specimen. The three color component images are registered monochrome images, each of which shows objects of a specific type. This paves the way for isolating, measuring, and classifying the objects by using standard techniques for the analysis of digital images.

Commonly, the absorption or emission spectra of the stains or fluors do not match the curves in Figure 1. Then the red, green, and blue primary colors of human vision (and of commonly available hardware) are not ideal for isolating the differently colored objects in the specimen. When it is necessary to display the specimen in its natural appearance, one must account for the spectral response of the camera and of the display unit in order to render an accurate-appearing reproduction. On the other hand, quantitative multispectral image analysis can be done using any set of (two or more) primary colors. In this case it is often advisable to abandon the RGB system and select primary colors that match the stains or fluors in use.

Color Balance

Often a digitized tri-color image will not render the various colors properly when displayed. Nonuniform illumination, combined with differing sensitivities and offsets (black levels) in the three color channels, can unbalance the three color-component images so that the objects in the field appear to be shifted in color. Most noticeably, objects that should be gray take on unwarranted color. The first test of proper color balance is that all the gray objects indeed appear gray. The second test is that the highly saturated (bright) colors must all have the proper hue (defined below).

The remedy for color imbalance is to use linear grayscale transformations (Castleman, 1996) on the individual red, green, and blue images. Normally, two of the component images are transformed to match the third, as follows. One selects two relatively uniform areas of the image, one light gray and one dark gray, and computes the mean gray level of both areas in each of the three component images. One then defines linear grayscale transformations (*i.e.*, $y = ax + b$) to make two of them (*e.g.*, red and blue) match the third (*e.g.*, green). One must solve two linear equations in two unknowns to determine a and b. This forces each of the two areas to have the same gray level in all three component images, and this will usually achieve color balance. If the camera and digitizing system are stable, the required transformations can be determined in advance by digitizing a black-and-white test target. The resulting transformations can then be used routinely throughout the digitizing session.

Color Specification

There are several ways to specify, quantitatively, the color of a pixel in a tri-color digital image. Here we discuss two of the more useful methods.

RGB format

The most common color specification simply uses the red, green, and blue brightness values scaled, for example, between zero and one. This convention is called "RGB format." The color of each pixel can be represented by the location of a point in the first quadrant of three-dimen-

sional color space (RGB-space). This is shown by the color cube in Figure 2.

The point at the origin of RGB-space represents zero brightness of all the primary colors and is thus the color black. Full brightness of all three primaries together appears as white at the corner diagonally opposite the origin of RGB-space. Three of the corners of the color cube correspond to the primary colors, red, green, and blue. The remaining three corners correspond to the secondary colors, yellow, cyan (blue-green), and magenta (purple). Equal amounts of all three color components at lesser brightness produce a shade of gray. The locus of all such points falls along the "gray line," which is the diagonal of the color cube connecting the black and white points (Fig. 2).

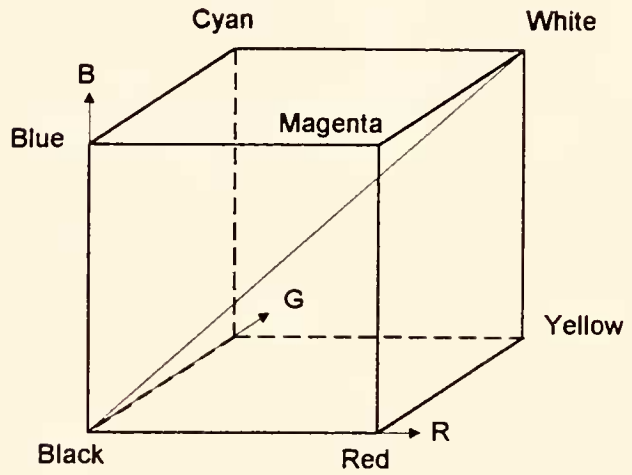

Figure 2. RGB color space. Each of the primary colors defines one axis of a three-dimensional coordinate system. Every color plots to a position in this space.

HSI format

Another useful specification scheme is called the "HSI format" (hue, saturation and intensity). It reflects the way humans perceive color, and it offers advantages for color image processing as well (Castleman, 1996; Pratt, 1991; Russ, 1995).

In HSI format the "I" stands for intensity, or brightness. It is, for our purposes, simply the sum of the R, G, and B gray level values, although different schemes with unequal weighting of the colors are also used. The intensity value specifies the overall brightness of the pixel, without regard to its color. One can convert a color image to monochrome by computing only the intensity at each point, thereby discarding the color information.

The color information is contained in two parameters that are called "hue" and "saturation," although other, equivalent terms are often used. Hue and saturation are

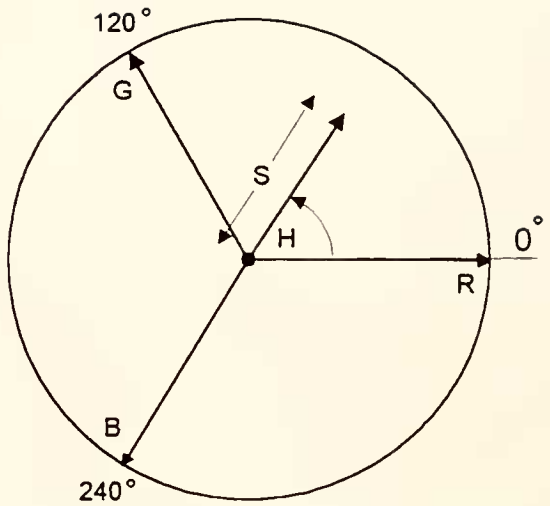

Figure 3. The color circle. Hue is an angle, and saturation is the radial variable.

illustrated by the color circle in Figure 3. The hue of a color refers to which spectral wavelength (color of the rainbow) it most closely matches, and is expressed as an angle. Arbitrarily, a hue of 0° is red, 120° is green, and 240° is blue. Hue traverses the colors of the visible spectrum as it goes from zero to 240 degrees. Between 240° and 360° fall the nonspectral (purple) colors that the eye perceives.

The saturation parameter is the distance of the point from the origin of the color circle. The "pure" or "saturated" colors fall around the periphery of the circle, and their saturation values are unity. At the center of the color circle lie the neutral (gray) shades; that is, those with zero saturation.

The concept of saturation can be illustrated as follows. Consider a bucket of bright red paint. The color has a hue of 0° and a saturation of unity. Mixing in some white paint makes the red less intense, thereby reducing its saturation, but without making it darker (*i.e.*, reducing its intensity). Pink corresponds to a saturation of 0.5 or so. As more white is added to the mixture, the red becomes paler, and the saturation decreases, eventually approaching zero (white). If, on the other hand, black paint is mixed with the bright red paint, the intensity of the color decreases (toward black) but its hue (red) and saturation (1.0) remain constant.

The three color coordinates, taken together, define a cylindrical color space (Fig. 4). The gray shades fall along the axis from black at the bottom to white at the top. The fully bright, fully saturated colors fall on the perimeter of the top surface of the cylinder.

Many other color coordinate systems are used. Those established by the Commission Internationale de l'Eclairage (CIE), an international standards committee for light and color, are perhaps the most widely used. They are

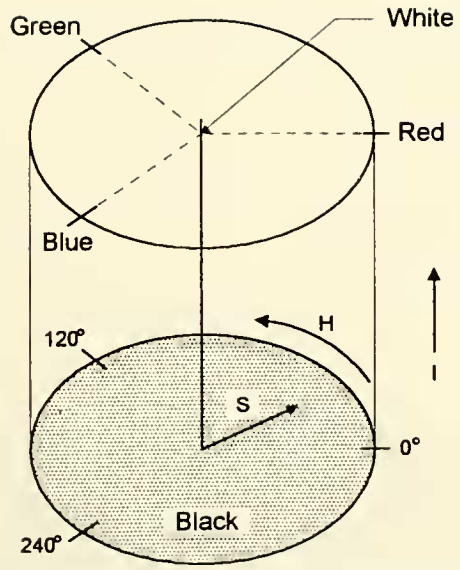

Figure 4. Cylindrical color space. Intensity is added as the axial variable. Every color plots to a position in this space.

based on experimental data from color-matching experiments conducted on human observers.

Color coordinate conversion

Some image processing techniques are intrinsically more successful when carried out in rectangular RGB-space, whereas others work best in cylindrical HSI-space. Thus it is useful to be able to convert color digital images from RGB to HSI color coordinates and back again.

RGB to HSI conversion

The conversion from RGB to HSI format can be approached as follows. Recall that the gray line is the diagonal of the color cube in RGB-space, and it is the vertical axis in cylindrical HSI-space. Thus we can begin by rotating the RGB cube in x, y, z-space so that its diagonal lies along the z-axis, and its R-axis lies in the x-z plane (Ledley *et al.*, 1990: Castleman, 1996). This is given by

$$x = \frac{1}{\sqrt{6}}[2R - G - B]$$

$$y = \frac{1}{\sqrt{2}}[G - B]$$

$$z = \frac{1}{\sqrt{3}}[R + G + B] = I \tag{1}$$

The latter of these gives the HSI intensity parameter.

We can convert to cylindrical coordinates by defining polar coordinates in the x-y plane. Then we normalize the radial variable so that the fully saturated colors (those having no more than two of the primary colors present) fall on a unit-radius circle in the x-y plane. This leads to the formula for saturation

$$S = 1 - \frac{\sqrt{3}}{I}\min(R, G, B) \tag{2}$$

We compute the angle

$$\theta = \cos^{-1}\left[\frac{\frac{1}{2}[(R - G) + (R - B)]}{\sqrt{(R - G)^2 + (R - B)(G - B)}}\right] \tag{3}$$

and the hue is

$$H = \begin{cases} \theta, & G \geq B \\ 2\pi - \theta, & G \leq B \end{cases} \tag{4}$$

Notice that black (*i.e.*, $[R, G, B] = [0, 0, 0]$) creates a problem for Eq. (3). We can assign black to $[H, S, I] = [0, 0, 0]$.

HSI to RGB conversion

The formulas for converting from HSI to RGB coordinates take on slightly different form for different sectors of the color circle (Castleman, 1996). For $0° \leq H < 120°$,

$$R = \frac{I}{\sqrt{3}}\left[1 + \frac{S\cos(H)}{\cos(60° - H)}\right]$$

$$B = \frac{I}{\sqrt{3}}(1 - S)$$

$$G = \sqrt{3}I - R - B \tag{5}$$

while for $120° \leq H < 240°$,

$$G = \frac{I}{\sqrt{3}}\left[1 + \frac{S\cos(H - 120°)}{\cos(180° - H)}\right]$$

$$R = \frac{I}{\sqrt{3}}(1 - S)$$

$$B = \sqrt{3}I - R - G \tag{6}$$

and for $240° \leq H < 360°$

$$B = \frac{I}{\sqrt{3}}\left[1 + \frac{S\cos(H - 240°)}{\cos(300° - H)}\right]$$

$$G = \frac{I}{\sqrt{3}}(1 - S)$$

$$R = \sqrt{3}I - G - B \tag{7}$$

The RGB cube (Fig. 2) does not map directly into the color cylinder of Figure 4. Instead, it defines a somewhat cone-shaped region in HSI-space, with white at the apex.

Since the entire floor of the color cylinder is the single color black, some authors prefer to consider the HSI color space to be biconic.

There are several variations of the HSI conversion formulas (Pratt, 1991), but the particular one chosen usually will not materially affect the result, as long as (1) intensity is a weighted average of R, G, and B; (2) hue is an angle; (3) hue, saturation, and intensity are independent; and (4) the transformation is accurately invertible.

Multispectral images having more than three primary colors can also be transformed into corresponding multi-dimensional color spaces. For example, one can convert from rectangular to spherical coordinates in a color space based on four or more primary colors.

Color Compensation

Available stains and fluorophores have different, often broad absorption and emission spectra, and commonly used color cameras have broad, overlapping sensitivity spectra. As a result, one seldom obtains complete isolation of three types of objects in the three component images.

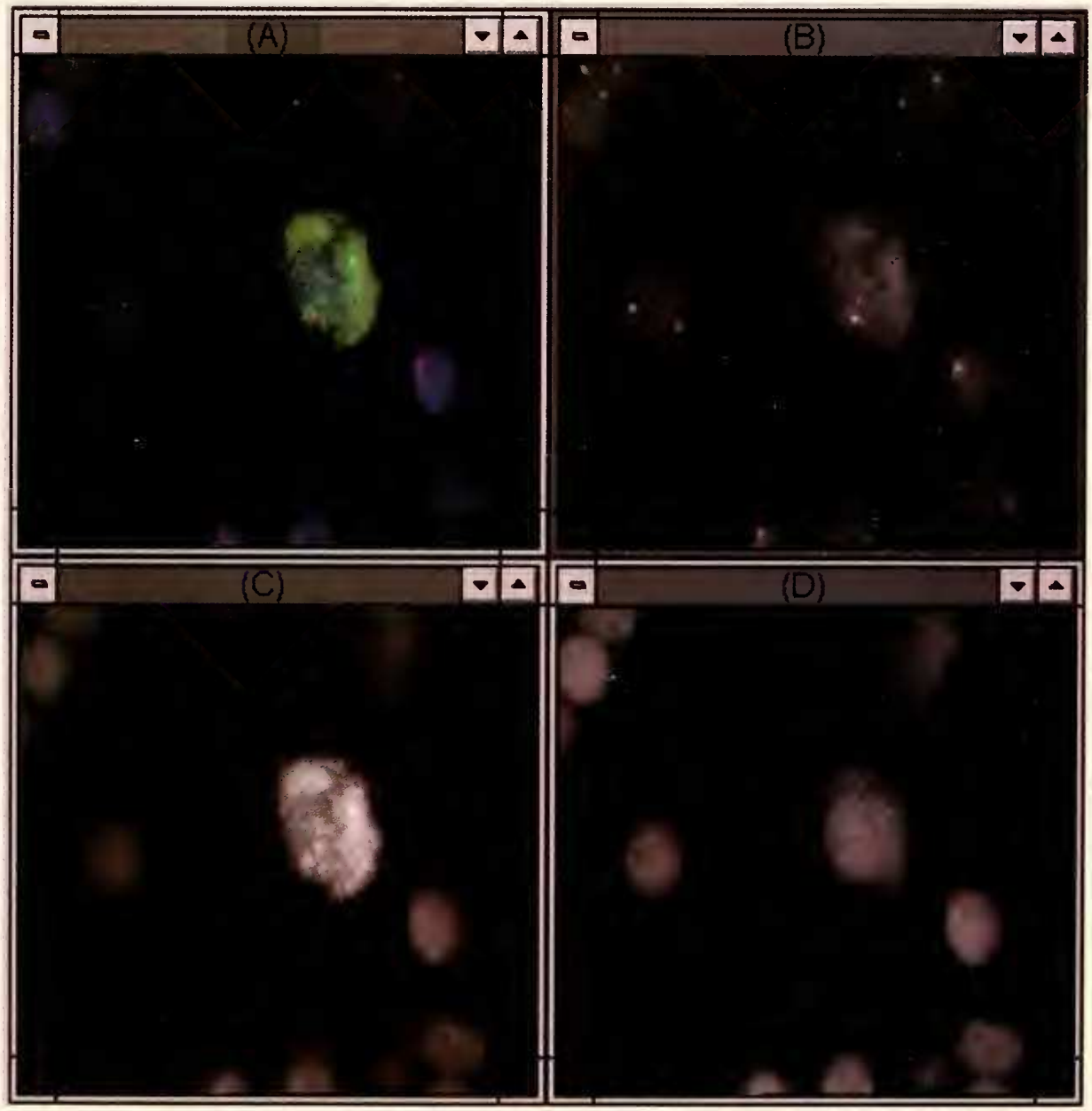

Figure 5. Three-color, fluorescently labeled bone marrow cells. (A) Color image; (B) red; (C) green; (D) blue. Notice that all structures appear in each of the R, G, and B images.

Instead, each type of object is visible in all three color-component images, although at reduced contrast in two of them (Fig. 5).

We can model the spreading of light among the color channels as a linear transformation (Castleman, 1993, 1994, 1996). Let the matrix, **C,** specify how the colors are spread among the three channels in a three-color fluorescence application (example below). Each element c_{ij} is the proportion of the brightness from fluorophore j that appears in color channel i of the digitized image. Let **x** be the 3 by 1 vector, $[R, G, B]^T$, of actual fluorophore brightness values at a particular pixel, scaled as gray levels that would be produced by an ideal color image digitizer (one with neither color spread nor black level offset). Then

$$\mathbf{y} = \mathbf{Cx} + \mathbf{b} \tag{8}$$

is the vector of RGB gray levels recorded, at that pixel, by the image digitizer. While **C** accounts for the color spread, the vector **b** accounts for the black level offset of the digitizer. That is, b_i is the gray level that corresponds to black (zero brightness) in channel i. Equation (8) is easily solved for the true brightness values by

$$\mathbf{x} = \mathbf{C}^{-1}[\mathbf{y} - \mathbf{b}] \tag{9}$$

Color spreading can thus be eliminated by first subtracting the black level from each channel, and then premultiplying the resulting RGB gray level vector for each pixel by the inverse of the color spread matrix.

Color compensation example

Figure 5 shows an RGB image of human bone marrow cells; the image was digitized with a single-chip CCD color television camera mounted on a fluorescence microscope. The camera's sensitivity spectra were similar to those in Figure 1. The cells were counterstained with DAPI (4′,6-diamidino-2-phenylindole, dihydrochloride), which fluoresces in the blue. Dividing cells also picked up the green fluorophore, FITC (fluorescein-5-isothiocyanate), which had been conjugated with BrdU (5-bromodeoxyuridine). The centromeres of the number 8 chromosomes were labeled with Texas red, a red fluorophore, attached to a DNA probe.

Ideally, the cells would appear in the blue channel, dividing cells would show up in the green channel as well, and two dots per cell (corresponding to the two number 8 chromosomes) would appear in the red channel. In Figure 5, however, all components appear in all channels because of overlap among the fluorophore emission spectra and the sensitivity spectra of the camera's three color channels.

Table I shows the color spread matrix for the system used to digitize the image in Figure 5. The values in this matrix were determined experimentally from digitized images of cells stained with single fluorophores. They represent the relative contrast of different colored objects in each of the three color channels. Table I states, for example, that only 44% of a DAPI molecule's brightness is recorded in the blue channel, while 32% of it shows up in the green channel, and 24% finds its way into the red channel. The color smear in this example is relatively severe, and use of a monochrome camera with separate, optimized, color filters, for example, can produce better results. But color smear is intrinsic to the imaging process and is always present to some degree.

Table I

Color spread matrix for the image in Figure 5

	Fluorophore		
Color channel	Texas Red	FITC	DAPI
Red	0.85	0.26	0.24
Green	0.05	0.65	0.32
Blue	0.10	0.09	0.44

The columns represent the three fluorophores used, and the rows represent the three channels of the color camera. The values in the table specify the proportion of the emitted light from each fluor that appears in each color channel.

The inverse, $\mathbf{C}^{-1}$, of the matrix in Table I is the color compensation matrix,

$$\mathbf{C}^{-1} = \begin{bmatrix} 1.24 & -.45 & -.35 \\ 0.05 & 1.69 & -1.26 \\ -.29 & -.24 & 2.61 \end{bmatrix} \tag{10}$$

It specifies how, using Eq. (9), one can correct the color spreading. The values in the left column specify that one should, at each pixel, take 124% of the gray level in the red channel image, add 5% of the green channel value, and subtract 29% of the blue value to produce a corrected red-channel gray level. The second and third columns specify how to correct the pixels of the green and blue channels, respectively.

Figure 6 shows the result of color compensation applied to the color image in Figure 5. Here the three differently stained types of objects are effectively isolated to the images in the red, green, and blue channels. This permits more accurate segmentation and measurement of the cell and its components. Because color spread tends to "wash out" an image, color compensation tends to increase both the contrast and the saturation of the displayed color image. Since it is intended to isolate the different colored components into separate color channels, it may not produce visually realistic images upon display.

Color Image Analysis

Image segmentation

Segmentation of a tri-color image can be viewed as a process of partitioning the three-dimensional (RGB or

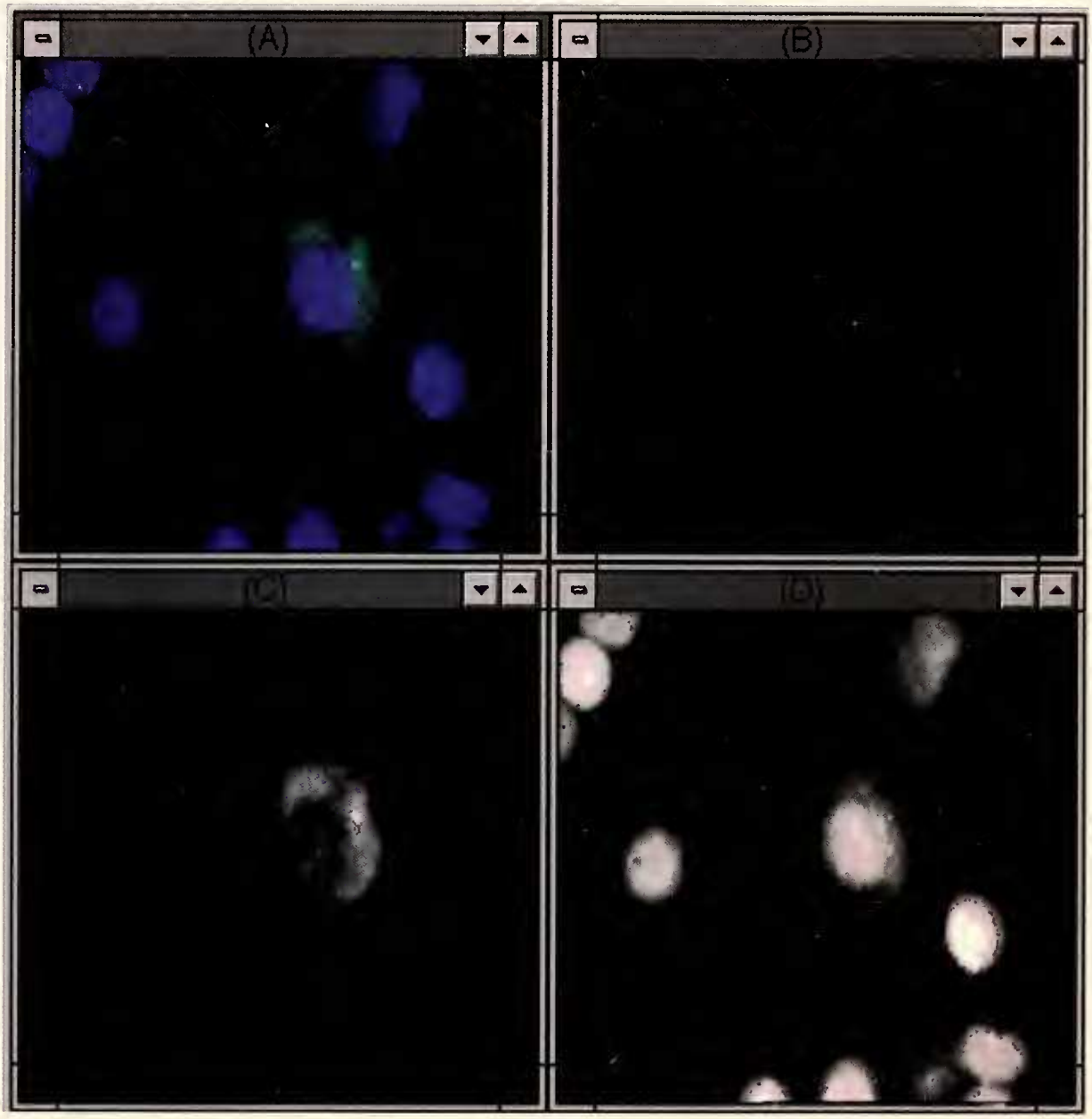

Figure 6. The effect of color compensation. This is the same image as Figure 5 after color compensation (Eq. (9)) was applied using the color smear matrix shown in Table I. (A) Color image; (B) red; (C) green; (D) blue. Notice that each of the different structures is isolated to the R, G, or B image.

HSI) color space. The different colored objects in the image correspond to separate clusters of points in color space, although the clusters corresponding to different objects may well overlap.

The hue and saturation of an object are normally dictated by the light-reactive properties of its dye or stain—how it absorbs, reflects, or emits light. The intensity, however, is seriously affected by illumination and viewing angle. Uneven illumination, for example, normally has much more effect on intensity than on the two color parameters. Thus it may be productive to segment the image in the hue-saturation plane (*i.e.*, on the color circle) rather than in three-dimensional color space, thereby ignoring intensity altogether.

Object measurement and classification

Once the objects in the image have been identified, they can usually be measured in one of the color-channel images. Sometimes two or more color-channel images can be combined to produce a monochrome image for analysis. Further, some objects may be present in different

channels and must be analyzed there. In any event, the measurement and classification of objects can usually be conducted with techniques that are commonly applied to monochrome digital images (Castleman, 1996; Pratt, 1991).

Discussion

The digital analysis of color microscopic images is becoming more important as new techniques for specimen preparation emerge. For example, each of the 24 types of human chromosomes can be painted with a distinct color by using multicolor fluorescent *in situ* hybridization (M-FISH) techniques (Speicher *et al.*, 1996, Schröck *et al.*, 1996). As the number of colors increases, and as the analytical requirements become more stringent, the demands placed on digital image analysis become more severe.

Fortunately the techniques mentioned here for tri-color images generalize readily to multispectral digital images with two, four, or more primary colors. Then the battery of techniques that have been developed for monochrome image analysis can be applied to the separate color-component images, after they have been balanced, compensated, and perhaps converted to another coordinate system.

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